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Detection of Chlamydophila abortus in sheep by Polymerase Chain Reaction

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This study was carried out on 180 pregnant ewes located at Ras Sedr research station - desert research center, from 2003 to 2005. Twenty five cases of abortion were recorded and examined serologically by complement fixation test (CFT). 17 (68%) out of these 25, showed positive results against *Chlamydophila abortus* and 3 (15%) out of 20 apparently healthy pregnant ewes were serologically positive. Due to the fact that both clinical signs and pathological findings are not specific in case of chlamydial infection and also due to the fact that CFT is accompanied by false positive reactions due to cross reactivity between chlamydial species, five samples from serologically positive aborted dams were subjected to polymerase chain reaction (PCR). They revealed positive results for *Chlamydophila abortus* at 119 bp. Therefore, PCR is proven to be reliable, rapid and specific diagnostic technique in the diagnosis of chlamydial infection.

Chlamydiosis is a contagious disease infecting all livestock species, but sheep and goats are the most commonly affected (Storz, 1971 and Sharma *et al*. 1983). The classic symptoms of the disease are abortion, still birth or premature delivery of weak lambs. Aborted animals are subsequently immune and will usually not abort with a chlamydial infection again. The animal is still infected and can transmit the organism to other animals through the placenta and vaginal discharges (Shalaby *et al*., 1987; Coetzer *et al*., 1994; Asrani *et al*., 1996, Batta *et al*., 1997; Radostits *et al*., 2000 and Rekiki *et al*., 2002).

Chlamydophila abortus (*Chlamydia psittaci*) is widely distributed obligate intracellular pathogens, which exhibits a broad pathogenic potential (Fukushi and Hirai, 1992).

Animals frequently become infected, but show no signs and stress may predispose clinical disease either as sporadic cases or as epidemics. The feco-oral is the most common route of transmission. Also aerosol, contact and venereal transmission are possible. Chlamydiosis may result in abortion, still birth or weakness of lambs and kids (Storz, 1971; Sharma *et al*., 1983; Miller *et al*., 1990 and Chiocco *et al*., 1992).

Chlamydia psittaci was found to be responsible for 20% of ovine abortion reported annually in Great Britain (Aitken, 1986) constituting the most common cause of abortion in sheep (Aitken *et al*., 1990). Abortion in newly infected flock with enzootic abortion may be as high as 30%, while the rate on flocks experiencing a reinfection is less than 5% (Kendrick and Howarth, 1992). The late abortion and premature lambing are the only clinical manifestations of enzootic abortion. Retention of the fetal membranes may occur in some cases (Jubb *et al*.,1993). *Chlamydia psittaci* was isolated from the placenta of the aborted ewes. Moreover, serological studies showed that Chlamydia antibodies in ewes' sera in Egyptian farms were 11.68% using complement fixation test (CFT) (El-Sayed, 1993).

CFT is the most widely accepted serodiagnostic method for chlamydial infection in animals (Kaltenbook *et al*., 1997) as it gives satisfactory results with ovine, caprine and avian serum samples, but not with bovine samples (Butty and Nicolet, 1987).

Pathogenic changes were observed as subcutaneous petechial hemorrhages in the skin of legs, hips, neck and in the head of chlamydial aborted foeti (Studdert, 1968). The necrotic placentitis is the primary pathological lesion of chlamydial infection in sheep and goats (Aitken, 1989).

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Table (1): The master mix ingredients and primers concentration used in PCR.

Constituent	Initial concentration	Amount (µl)	Final concentration	x^8
Distilled water		13.2		105.6 µl
Buffer	10 x	2.0		16.0 µl
dNTP	10mM	0.4	0.2mM	3.2 µl
Taq polymerase	5µ/Ml	0.4	2µ/Ml	3.2 µl
Primer 2AF	20 mM	1.0	1 mM	8.0 µl
Primer 2Br	20 mM	1.0	1mM	8.0 µl
Total volume		18.0 µl		144.0 µl

Polymerase chain reaction (PCR) is one of the most modern advanced techniques used for accurate diagnosis of the causative agents (Creelan and McCullough, 2000; Amin, 2003). It has rapidly become one of the most widely used techniques in molecular biology. It was proven to be rapid, relatively inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of DNA material.

The aim of this study was to investigate the presence of Chlamydial infection by PCR and serodiagnostic studies using complement fixation test. Histopathological changes were also studied.

Materials and Methods

Animals. This study was performed on total number of 180 pregnant ewes located in Ras Sedr Research Station (this station belonged to Desert Research Center). History and clinical examination of animals were recorded. Samples were collected during the period from 2003 to 2005

Serum samples. They were collected from aborted ewes (4 weeks post abortion) as well as from apparently healthy pregnant ewes for detection of chlamydial antibodies using complement fixation test.

Tissue samples. Tissue samples were collected from placenta of aborted ewes and internal organs of aborted foeti and newly born deaths (liver, kidneys, heart, brain, lungs and spleen) for gross examination and histopathological studies. The collected samples were fixed in 10% neutral buffered formalin. The fixed specimens were then washed, dehydrated and embedded in paraffin wax. The tissues were sectioned at 4-5µ thickness and stained with haematoxylin and eosin (H&E) for histopathological examination and stained with Gemeniz stain as special stain for *Chlamydia psittaci* (Bancroft *et al*., 1996).

Antisera. Reference antisera for chlamydia (*Chlamydia psittaci* CFT reagents, " Seiken "), were obtained from Denka Seiken Co., Tokyo, Japan. Anti-sera were used for detection of chlamydial antibodies in the suspected materials.

Reference Chlamydial antigen. Obtained from Denka Seiken Co., Tokyo, Japan. It was used in serological detection of antibodies.

Complement. Freeze dried preparation of preserved guinea pig serum (Welcome) was used in Complement Fixation Technique.

Polymerase Chain Reaction (PCR). From (5) serologically positive cases for chlamydiosis, tissue samples (placenta, internal organs of aborted foeti as liver, kidney, lung and brain) were subjected to PCR. Deparaffinizing the paraffin embedded samples then starts the process of DNA extraction.

DNA extraction. The genomic DNA was extracted from samples using Dneasy tissue kit purchased from QIA Gen, Basel, Switzerland according to (Venables *et al*., 1997).

PCR amplification of chlamydial DNA. It was performed on the extracted DNA from tissue samples using oligonucleotide primers Chla.2 AF:5-GCTTTTCTAATTTACACC-3 and Chla. 2 Br: 5- ATAGGGTTGAGACTATCCACT - 3 according to (Sykes *et al*., 1997). 2 µl of template added to each tube and 2 µl of distilled water added to tube of negative control (Table. 1). The reaction was subsequently run at 95°C for 10 min. then for 40 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, followed by an additional elongation at 72°C for 10 minutes. Reaction product was visualized by ethidium bromide staining under UV transillumination after electrophoresis on 1.5% agarose gel.

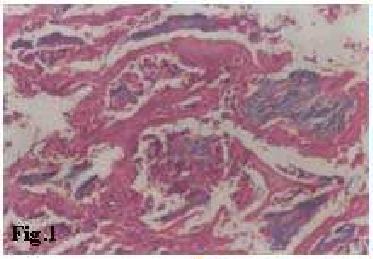
Results

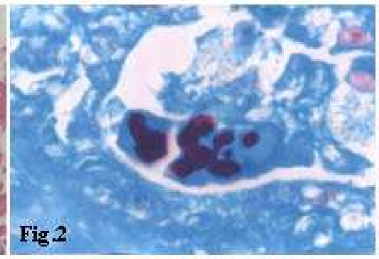
History of farms. The rate of abortion in the first year (2003) was high in pregnant ewes, while the rate of abortion decreased year after year.

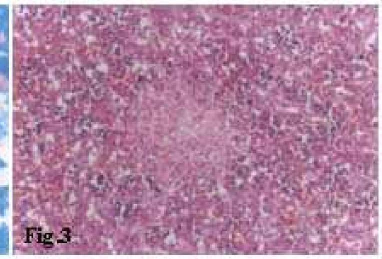
Table (2): Results of CFT in aborted and apparently healthy pregnant ewes.

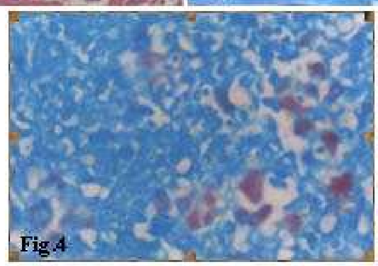
Pregnant ewes	No of aborted ewes			No of samples of apparently healthy pregnant ewes		
Results of CFT	No	Positive	%	No	Positive	%
	25	17	68	20	3	15

CFT titer ranged from 1/8 – 1/128. Positive sample more than 1/32.









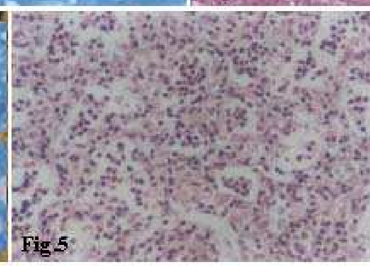


Fig. (1): Fetal placenta, showing severe extensive necrosis, myxomatosis degeneration of chorioallantoic villi and presence of inflammatory cells and cellular debris within the crypts (H & E, x 100).

Fig. (2): Fetal placenta, showing the presence of chlamydial elementary bodies within the trophoblast cells. (Gimenez stain, x 1000).

Fig. (3): Liver of aborted foetus, showing focal area of hepatic cell necrosis associated with diffuse infiltration of lymphocytic cells (H & E, x 100).

Fig. (4): Liver of aborted foetus, showing the presence of intracytoplasmic chlamydial elementary bodies as bright granules (Gimenez stain, x 400).

Fig. (5): Lung of aborted foetus, showing aggregations of neutrophils and macrophages within the alveoli (H & E, x 200).

Clinical manifestation. The most common clinical signs observed in pregnant ewes were abortion at late stage of pregnancy in the first year, while still birth or birth of weak unthrifty lambs were recorded in the second and third year more than abortion.

Serological studies. Results of serological studies were demonstrated in Table 2.

Histopathological findings:

Fetal placenta. There was severe necrosis of chorioallantoic villi with sloughing of the trophoblastic cells covering the villi into the Severe haemorrhages in the intercotyledonary areas were seen. Marked signs of vasculitis were noticed. Chlamydial elementary bodies were detected in the cytoplasm of trophoblsats as red

Aborted fetal organs.

Liver. It showed multiple foci of hepatic cell necrosis associated with diffuse infiltration of mononuclear cells aggregations mostly lymphocytes in hepatic parenchyma (Fig. 3). It appeared as bright red granules against blue back ground in section stained with Gimenez stain (Fig. 4).

Lung. It displayed aggregations of neutrophils and macrophages in the lumen of alveoli (Fig. 5). In addition, the alveolar walls were thickened, pulmonary blood vessels appeared dilated and congested.

Results of PCR. Five randomly collected

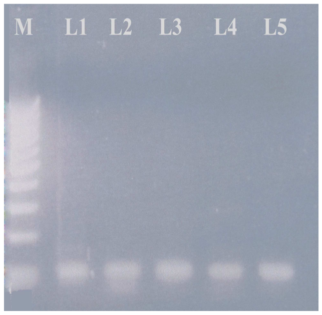


Fig. (6): PCR of (*Chlamydiophila abortus*) DNA from formalin-fixed and parafin embedded tissues of aborted ovine foeti. PCR products were separtaed on 1.5% agarose gel and stained with ethidium bromide M, 100 bp ladder marker; lane 1-5 specific *C. psittaci* PCR product (119 bp detected).

samples of placenta and aborted foeti of aborted ewes from serologically
positive cases for *Chlamydophila abortus* revealed positive results by using PCR (119 bp). The positive control showed the excepted amplification product (119bp) as shown in Fig. (6).

Discussion

Ovine chlamydial abortions (ovine enzootic abortion) is an infectious and contagious disease clinically characterized by abortion and weak neonates. The etiological agent is *Chlamydophila abortus* (*Chlamydia psittaci*) which is considered one of the most common pathogens of small ruminants. Chlamydiosis is the principle cause of infertility in bovines as well as in small ruminants (Pugliese, 2001). It has important economic implications and represents a significant zoonotic risk. Clinical diagnosis is often difficult because the clinical and pathological lesions are not specific (Marsilio *et al*., 2005).

A definitive diagnosis is based on correlation between both clinical signs and pathological findings. The obtained results were identical to those described previously by (Eisa and Hamoda, 2002; Kendrick and Howarth, 1992).

Serological studies using complement fixation test is the most widely used serological test for detection of chlamydiosis. Positive results were detected in 17 (68%) out of 25 of aborted cases and 3 (15%) out of 20 of apparently healthy pregnant ewes. The detection of chlamydial antibodies in investigated samples by CFT was identical to that obtained by Schmatz *et al*. (1978); Martin (1995); Mousa *et al*. (1998); Joshi, (1998); Ozturic, *et al.* (1998); Vojinovic (1999) and Quinlan (1999).

CFT is sensitive test as it can diagnose *Chlamydia psittaci* in 20% of sera of aborted ewes (Duman and Durak, 1998).

Seroprevalence survey of chlamydiosis among cows and buffaloes using CFT, agar gel precipitation test and elementary body agglutination test resulted in positive percentages of 21.16%, 17.5% and 12.5%, respectively and the CFT was the most sensitive test (Paul *et al*., 2002). In addition, Buendia *et al*. (2001) used CFT in diagnosis of *Chlamydophila abortus* in sera of sheep with 71% sensitivity and 83.6% specificity while Cislokova *et al*. (1999) diagnosed *C. psittaci* in small mammals by CFT with 16.9% positivity, it is concluded that small mammals play an important role in transmission *C. psittaci*.

On the other hnad, ELISA is more sensitive and specific than CFT and can detect chlamydial antibodies in sera of sheep (Borel *et al*., 2002; Henning and Sting, 2002) as CFT is complicated by false positive reaction resulting from cross reactive antibodies to *Chlamydophila pecorum* (Longbotton *et al*., 2001).

The main histopathological findings in the placenta and aborted foeti of aborted ewes due to chlamydiosis were development of necrosis and inflammatory changes in internal organs. These results were parallel with that of Buxton *et al*. (1990; 2002); Chanton *et al*. (2002) and Desouky *et al*. (2004). Such changes could be attributed to embolic dissemination of chlamydial infection from placenta (Buxton *et al*., 1990) as indicated by the presence of elementary bodies in the liver of aborted foeti. The initial interaction of Chlamydia with the host cells begins with the attachment of elementary bodies to the cells followed by phagocytosis within membrane limited vacuole called inclusion which don't fuse with lysosomes of cells and explain the survival of the organism in the intracellular environment (Escalante-Ochoa *et al*., 1998).

It is interesting to mention that clinical signs and pathological findings are not specific also, complement fixation test is complicated by false positive a fact that necessitate the use of another sensitive test like PCR in the diagnosis of chlamydiosis.

Five randomly collected tissue samples of placenta and aborted foeti, (serologically positive for *C. psittaci*) were subjected to PCR using 2A and 2B primers which are specific for

identification of *C. psittaci* DNA. All the examined tissue samples showed the expected amplification product specific for *C. psittaci* (119 bp). These findings were in parallel with (Thiele *et al*. 1992; Creelan and McCullough 2000; Mi-Zu Huang *et al*., 2002; Amin 2003; Desouky *et al*., 2004) who reported that PCR is a specific, sensitive and rapid technique for detection of *Chlamydophila abortus* (*C. psittaci*) in ewes. Also with PCR it is possible to diagnose *C. abortus* from archival material with no culture examination. Furthermore, PCR is less labour consuming and the results can be obtained within a few hours.

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الكشف على الكلاميدوفيلا ابورتس فى الاغنام باستخدام اختبار تفاعل البلمرة المتسلسل

أجريت هذه الدراسة على عدد ١٨٠ نعجة عشار فى محطة رأس سدر التابعة لمركز بحوث الصحراء من سنة ٢٠٠٣ حتى سنة ٢٠٠٥ وقد حدثت عدد ٢٥ حالة بنسبة ١٣,٨٨% (من عدد١٨٠ نعجة) إجهاض فى الثلاث سنوات خضعت للاختبار السيرولوجى تثبيت المتمم بعدد ١٧ حالة ايجابية بنسبة (٦٨%) من إجمالى حالات الإجهاض لميكروب الكلاميدوفيلا ابورتس ، وكذلك تم اختبار عدد ٢٠ عينة سيرم من النعاج العشار السليمة ظاهريا حيث ظهرت عدد ٣ حالات ايجابية بنسبه (١٥%) ولما كانت الأعراض الإكلينيكية والتغيرات الباثولوجية غير دقيقة لتشخيص العدوى بالكلاميديا وكذلك الايجابية الراجعة للتداخل الوارد فى اختبار تثبيت المتمم نتيجة التداخل بين عائلة سلالات الكلاميديا فقد تم عمل اختبار تفاعل البلمرة المتسلسل (PCR) لعدد خمس أجنة مجهضة لحالات ايجابية سيرولوجيا وكانت النتيجة ايجابية لميكروب الكلاميدوفيلا ابورتس عند وزن جزيئي bp ١١٩ . ولهذا يعتبر اختبار تفاعل البلمرة المتسلسل سريع ومتاح ودقيق فى تشخيص عدوى الكلاميديا .